Reference intervals for hematological parameters in wild Eastern grey squirrels (*Sciurus carolinensis*)

Claudia Romeo [1]†, Sara D'Alessandro [1], Mattia Bielli [2], Stefano Comazzi [1] and Nicola Ferrari [1]

[1] Department of Veterinary Medicine, Università degli Studi di Milano, Milano, Italy

[2] Private Practitioner, Novara, Italy

†corresponding author: claudia.romeo@unimi.it

ORCID IDs:

Claudia Romeo: 0000-0002-5964-2847

Stefano Comazzi: 0000-0002-1567-8055

Nicola Ferrari: 0000-0001-8959-3495

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Abstract

The eastern gray squirrel (*Sciurus carolinensis*) is a North American tree squirrel species which has been introduced to several countries outside its native range. We made use of squirrels culled in Italy within control activities to establish hematological reference intervals for this invasive species, with the aim to broaden the current knowledge about its physiology. Blood from 104 individuals was analysed through a laser analyzer. Manual leukocyte differential counts on blood films were performed on a subset of 52 animals for validation of the automated counts, resulting in a good agreement between the two methods. Reference intervals and the 90% confidence interval of their limits were estimated for 19 hematological parameters, partitioning by sex when statistically significant differences were detected. For most variables, our results were comparable with the few data available on the grey squirrel and on related species. This study gathered valuable reference information for studies aimed at disclosing the mechanisms underlying grey squirrels' invasiveness and for future research on tree squirrels physiology.

Keywords: *Sciurus carolinensis*; hematology; reference values; blood parameters; tree squirrels

Introduction

The Eastern grey squirrel (*Sciurus carolinensis*) is a tree squirrel species native of eastern North America, which has been introduced in the British Isles, Italy and South Africa (Bertolino et al. 2014). In their native range, grey squirrels commonly inhabit temperate broad-leaved forests, urban parks and gardens across a wide latitudinal range and are hunted or subjected to pest control in many areas (Koprowski 1994). In the European Union, the rodent is currently included in the List of Invasive Species of Union Concern (EU Regulation 1143/2014) due to its documented invasiveness and impact on human activities and native ecosystems (Tompkins et al. 2003; Gurnell et al. 2004; Romeo et al. 2015; Santicchia et al. 2018). Nowadays, broadening our knowledge about biological characteristics of invasive species is fundamental to achieve a better understanding of the mechanisms underlying their invasiveness and their potential as pathogens reservoirs (Hulme 2014; Dunn and Hatcher 2015). However, as it often occurs for wildlife, information about invasive species physiology is often lacking or sparse. To our knowledge, only a handful of studies reported hematological values of grey squirrels and specific reference intervals are lacking (Guthrie et al. 1966; Hoff et al. 1976; Barker and Boonstra 2005; Yoder et al. 2011). Within a broader and ongoing project aimed at defining the epidemiological role of this invasive rodent in Italy and the mechanisms behind its invasiveness, the present study is therefore aimed at defining hematological reference intervals for the species, making use of a large sample of animals obtained from invasive species control activities.

Materials and Methods

Sampling

The grey squirrels sampled for this study were culled from September 2017 to May 2018 in NW Italy, within an invasive species control program carried out under the approval of the designated national institution (Italian Institute for Environmental Protection and Research, ISPRA) and with permits by the authorities for wildlife research and management of Città Metropolitana di Torino and Provincia di Cuneo (authorization nr. 62-3025 of 2017 and Prot. nr. 2016/98585 of 29/12/2016, respectively). The animals were captured using live-traps (mod. 202 Tomahawk Live Trap Co., WI, USA) that were checked at least twice a day, in order to reduce containment stress. Detailed information about trapping and handling procedures is available elsewhere (e.g. Santicchia et al. 2019). Once trapped, the individuals were humanely euthanized on the field by placing them in chambers pre-charged with 100% CO_2, in compliance with the European

Commission and the American Veterinary Medical Association guidelines (Close et al. 1996, 1997; Leary et al. 2013). Blood samples were collected through post mortem intracardiac puncture right after euthanasia (about 2.5 ml/individual). For each animal, 1 ml of blood was stored in ethylenediamine tetraacetic acid (EDTA) blood collection tubes, the remaining portion was stored separately for sera separation. All the samples were stored at 4°C within 2 hours from collection and until laboratory analysis. Fecal samples and carcasses for parasitological and necroscopic examination were collected for other studies.

Hematology

A complete blood count (CBC) and a leukocyte differential count (LDC) were performed within 48 hours from blood collection using a Sysmex XT-2000iV hematology laser analyzer (Sysmex Corporation, Kobe, Japan). Species-specific regions were drawn on the differential scatterplot in order to identify the clusters of neutrophils, eosinophils, lymphocytes and monocytes. Most hematology analyzers cannot reliably detect basophils, hence automated counts were not considered in further analyses and in reference intervals estimation (e.g. Lilliehöök and Tvedten 2009, 2011). On a subset of 52 samples for which we had automated LDC, blood films were made from the EDTA portion and stained 6 minutes in May-Grunwald and 30 minutes in Giemsa diluted 1:20 in distilled water. Manual leukocyte differential counts were performed with a microscope (ZEISS Primo Star), using 400x optic lens and 1000x in oil immersion. Four films were discarded due to low quality.

Statistical analysis

For the estimation of reference intervals, we followed the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) and the American Society for Veterinary Clinical Pathology (ASVCP), as described by Friedrichs et al. (2012). Firstly, the distribution of each hematological variable was tested for normality through Shapiro-Wilk tests and potential outliers were identified following Tukey's method, applying Box-Cox transformation on non-normal variables. Suspected outliers were individually inspected and excluded from the reference dataset when considered aberrant observations. Subsequently, we explored each variable for sex- or age-related differences by either t tests or Mann-Whitney tests. On all the variables that differed between sexes we estimated partitioned intervals, while when an age effect was detected the values from subadult squirrels were removed from the dataset, as their sample size (n=17) was too small to estimate a specific interval (Friedrichs et al. 2012). Reference intervals of most variables were obtained through the nonparametric method, applying bootstrapping techniques for the estimation of the 90% confidence interval (CI) of their limits. When sample size was lower than 80, the robust method was used. Finally, on the subset of grey squirrels for which blood films were available, we assessed the agreement between automated and manual LDCs through Bland-Altman plots and limits of agreement estimation (Bland and Altman 2003). Estimation of reference intervals and their 90% CIs was carried out using Reference Values Advisor 2.1 Software (Geffré et al. 2011). All the other analysis were carried out using SAS 9.4 Software (Copyright © 2012 SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Blood was sampled from a total of 113 grey squirrels: 93 adults (54 females and 39 males) and 20 subadults (i.e. <1 year old, 14 females and 6 males). Of these, 43 individuals were trapped during spring, 34 during autumn and 36 in winter. Post mortem examination of carcasses and fecal examinations carried out for other research revealed that the animals were in good condition, with no clinical signs of disease and only mild ectoparasitic and endoparasitic infections, in agreement with previous findings on the same metapopulation (Romeo et al. 2014a, c, b, 2018, 2019; Hofmannová et al. 2016; Prediger et al. 2017; Santicchia et al. 2019). Examination of blood films (n=52) did not show any hemoparasitic infection, but revealed the presence, among granulocytes, of neutrophil-like cells characterized by an hypersegmented nucleus, stained deep purple/blue with abundant eosinophilic granules scattered through the cytoplasm (Fig. 1b). Hypersegmented neutrophils have been reported in the congeneric Persian squirrel (*S. anomalus*, Asadi et al. 2007; Khazraiinia et al. 2008) and are consistent with descriptions of heterophils reported in other rodents and lagomorphs (Campbell and Ellis 2013). Two basophils, which are rarely reported in grey squirrels and in related species (e.g. Barker and Boonstra 2005; but see Hoff et al. 1976), were observed on two different smears (Fig. 1f).

All the hematological variables were distributed normally, except for the leukocytes (both total counts and LDC) and the mean corpuscular hemoglobin concentration (ESM 1: Supplementary Fig. 1). A total of 57 out of 1844 values in the initial dataset were identified as outliers and excluded from the reference dataset (range: 0 –7 removed values per variable). Final sample size, mean (± standard error), range and coefficients of variation (CV) of CBC and LDC following outliers' removal are reported in ESM 1 (Supplementary Table 1). Inter-individual variation for red blood cells parameters was limited, ranging from 3.9% CV in mean corpuscular hemoglobin to 8.7% CV in hematocrit, whereas a higher variability was recorded for platelet counts (24% CV) and platelet-related parameters. Even after outliers removal, inter-individual variation in white blood cells remained high: total leukocytes had a 45.6% CV and differential counts ranged from 64.7% CV for monocytes to 89.9% CV for eosinophils.

Estimated reference intervals for CBC parameters and LDC (absolute values obtained from the laser analyzer) are reported in Table 1. Red blood cell counts, hemoglobin and hematocrit varied depending on squirrel sex, with higher values in males than females (all $p<0.035$). Male-biased red blood cells parameters are observed in many mammalian species and are probably related to sex-hormones (e.g. Murphy 2014). Similar results on a small sample of grey squirrels were indeed obtained by Yoder et al. (2011). Additionally, these same variables plus the platelecrit were significantly higher in adult than subadult squirrels (all $p<0.022$), whereas the latter had higher mean corpuscular hemoglobin values ($p=0.031$). Sex- or age-related differences in leukocyte types were detected only in lymphocytes (higher counts in females than males, $p=0.025$) and neutrophils (higher counts in adults than subadults, $p=0.038$).

The Bland-Altman plots showed a fairly good agreement between the LDC (%) obtained through the laser analyzer and through blood film examination. In particular, the mean differences in neutrophils, lymphocytes and eosinophils counts estimated with the two techniques were close to zero, and limits of agreement were acceptable (ESM 1: Supplementary Table 2, Supplementary Fig. 2). Conversely, monocytes were virtually absent on blood films, resulting in some disagreement between the automated and the manual percentage. The overestimation of monocytes and the concurrent slight underestimation of lymphocytes by the laser analyzer suggests that the instrument may misclassify as monocytes other mononuclear cells.

As already mentioned, only a few studies reported hematological values in the grey squirrel (Guthrie et al. 1966; Hoff et al. 1976; Barker & Boonstra 2005; Yoder et al. 2011), but in most cases the mean values (for both CBC and LDC parameters) reported by these authors fall within the reference intervals estimated by the present study. An exception is represented by the hematocrit, for which we obtained slightly higher values than those reported by Barker and Boonstra (2005) and Hoff et al. (1976). Complete data about red blood cells volume (MCV) and hemoglobin content (MCH and MCHC) in grey squirrels were available only in Barker and Boonstra (2005; N=12) and in this case the squirrels in our sample appear to have bigger erythrocytes with a lower hemoglobin content per cell. Our results were also comparable to values reported for the congeneric Caucasian squirrel (Asadi et al. 2007; Kharzaiinia et al 2008). To our knowledge, no information about the hematological parameters of Eurasian red squirrels (*S. vulgaris*) is available for comparison.

Estimating reference intervals for wild species can be challenging due to small sample sizes, alteration of parameters following containment stress and a lack of knowledge about the actual health and physiological status of sampled individuals. Here, we made use of a large reference sample obtained through culling activities and by using an euthanasic method which has been previously shown not to alter hematological values in laboratory rodents (Walter 1999; Pecaut et al. 2000). When we defined the reference dataset, we also took into account information about individual clinical and parasitological status collected for other studies through PM examination. However, it must be noted that although the applied trapping protocol was aimed at minimising capture stress, animals were briefly handled prior to euthanasia and this might have induced an alteration of hematocrit (see Desantis et al. 2016 on flying squirrels, *Glaucomys* spp.), neutrophils and/or lymphocytes values (see Delehanty and Boonstra 2009 on ground squirrels, *Spermophilus richardsonii*).

Despite these limitations, the estimated intervals may be used as standard profiles in studies aimed at disclosing the mechanisms underlying grey squirrels' invasiveness. Additionally, this study gathered valuable reference information for future research on tree squirrels physiology, which still remains poorly studied compared to other rodent groups.

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed as detailed in Materials and Methods section.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure captions:

Fig. 1 White blood cells of grey squirrels (*Sciurus carolinensis*): a) band neutrophil and b) hypersegmented neutrophil; c) lymphocyte; d) monocyte; e) eosinophil; f) basophil (upper left) and hypersegmented neutrophil (bottom right).